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In vitro studies on tumor-specific immunity by using C3H mammary cancer-A cells. II. Specificity in growth-inhibition effect of lymph-node cells from sensitized animal on target cells*

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Abstract

For purpose to study specificity in the growth inhibition effect of sensitized lymph-node cells on target cells, the regional lymph-node cells obtained from the truly isologous mouse previously inoculated with A strain cells (derived from C3H mouse mammary cancer) were cultured with A cells in various ways, and obtained the following results. 1. Those regional lymph-node cells from the isologous mice transplanted with the skin of C3H mouse or MC-induced sarcoma do not inhibit the growth of A cells in tissue culture. 2. The regional lymph-node cells from the mice positive to tuberculin test also do not inhibit the growth of A cells in tissue culture. 3. The regional axillary lymph-node cells (C3H anti-A strain cells) inhibit the proliferation of M cells from Cb mouse mammasy cancer and JTC-ll cells from Ehrlich ascites tumor as well as A cells. However, these axillary lymphnode cells do not inhibit the growth of AH-66F cells from rat DAB hepatoma, Hela-S3 cells from human uterine cancer and L cells from subcutaneous connective tissue of C3H mouse. From these results it is assumed that the sensitized regional lymph- node cells act specifically on cancer antigen.

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IN VITRO STUDIES ON TUMOR-SPECIFIC IMMUNITY BY USING C3H MAMMARY CANCER-A CELLS
II. SPECIFICITY IN GROWTH-INHIBITION EFFECT OF LYMPH-NODE CELLS FROM SENSITIZED ANIMAL ON TARGET CELLS

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In the previous paper it was reported[1] that in the transplantation of A strain cells (derived from C3H mouse mammary cancer) to isologous mice regional lymph-node cells from the host showed at a certain early stage after the transplantation an inhibitory effect on the proliferation of A strain cells in tissue culture, and it was deduced that this phenomenon was attributable to the sensitization of the regional lymph-node cells by cancer antigen. For the clarification of the problem whether such an interaction between cancer cells and lymph-node cells *in vitro* is a reflection of immunological reactions or not, it is necessary to determine whether there is specificity in such reaction. For this purpose a series of experiments was conducted to see whether regional lymph-node cells from the isologous mice transplanted with skin graft of C3H mouse or methylcholanthrene-induced sarcoma or lymph-node cells from the mice positive to tuberculin test, will inhibit the proliferation of A strain cells. Concurrently, a study was made to see whether or not the regional lymph-node cells from the C3H mice transplanted with A strain cells would inhibit the growth of homologous and heterologous tissue culture cells. As the result it was found that in the interaction between antigenic cells and regional lymph-node cells *in vitro* there is a considerable specificity, and this is probably due to the specific action of regional lymph-node cells on cancer antigen. This report describes principal findings of such a study.

MATERIALS AND METHODS

Animals : Inbred C3H female (H-2^k) mice of age range, 6～8 weeks old, were divided into three large groups.

Antigens for sensitization : Skin graft of full thickness from C3H mouse, methylcholanthrene-induced sarcoma in C3H mouse, and A strain cells derived from C3H mouse mammary cancer served as antigen. To obtain tuberculin-

positive mice BCG in Freund's adjuvant was used. For the control groups complete and incomplete Freund's adjuvants were used.

Sensitization of mice and preparation of lymph-node cell suspensions : First group is divided into two subgroups, and with one subgroup the skin graft 1.2cm in diameter is transplanted to isologous C3H mice on the back between the scapulas by the method of BILLINGHAM and MEDAWAR[2], and axillary lymph nodes are taken out 9 days after the transplantation. To the other subgroup three slices ($1mm^3$) of methylcholanthrene-induced sarcoma are transplanted to isologous under the skin on the back between the scapulas and regional lymph nodes are removed on about 14 days afterwards when the tumor has grown to the size about 1 cm in diameter.

The regional lymph nodes from respective subgroup are cut into small pieces with ophthalmic scissors, passed through 80-mesh filter, washed three times by centrifugation at 2, 000 rpm for 5 min each in cold Hank's solution, and after removing serum, the cells are suspended in the 50% bovine serum plus YLE medium and this serves as the lymph-node cells suspensions. These suspensions are named as iso-L and MC-L respectively.

The second group is divided into three subgroups, and to one group 1 ml of complete Freund's adjuvant mixed with 0.5 mg BCG is injected subcutaneously on the back, once a week later 0.5 ml BCG is injected into the tail vein, two weeks afterward, confirming the animals to be positive to the tuberculin test in the skin, axillary lymph nodes are taken out. To the second and third subgroups 1 ml each of complete and incomplete Freund's adjuvant is injected subcutaneously, and axillary lynph nodes are taken out three weeks later. Cell suspensions are prepared in the same manner as in the first group, and each of these suspensions is designated B-L, c-L and i-L respectively.

To the third group 5×10^6 A strain culture cells derived from C3H mouse mammary cancer are transplanted snbcutaneously on the back, and on the tenth day after the transplantation regional lymph nodes are taken out. Cell suspension is prepared in the same way as in the first group and it is designated A-L.

With every group axillary lymph nodes of untreated normal C3H mice serve as control, and the lymph-node cell suspension prepared with them is n-L.

Tissue culture cells : 1) A strain culture cells derived from C3H female mouse ($H\text{-}2^k$) mammary cancer ; 2) L cells, derived from subcutaneous connective tissue of C3H mouse ; 3) M strain culture cells, mammary cancer cells derived from Cb femals mouse ($H\text{-}2^d$) ; 4) JTC-11 strain cells, derived from Ehrlich ascites tumor ; 5) AH-66F strain cells derived from rat DAB hepatoma ; and 6) HeLa-S_3 cells derived from human uterine cancer. Of these six cell strains, A-cells, M cells and HeLa-S_3 cells are at first treated with 0.25% trypsin GKN solution and A cells and M cells are suspended in 50% bovine

serum+YLE medium. JTC-11 cells and AH-66F cells are detached gently from the wall of test tube with a rubber cleaner and by adding 20% bovine serum +YLE medium, the cell suspension is prepared. L cells were cultured by a similar method in 10% bovine serum+YLE medium.

Culture of lymph-node cells and tissue culture ccls :

Experiment 1: Iso-L and MC-L lymph-node cell suspensions of the first group are mixed with A-strain cells and cultured in the medium optimal to A cells at 37°C.

Experiment 2: B-L, c-L, i-L lymph-node cell suspensions of the second group are each mixed with A-cells and cultured in the identical medium in the same way.

Experiment 3: In the third group A-L lymph-node cell suspension is mixed with A cells, L cells, M cells, JTC-11 cells, AH- 66F and HeLa-S_3 cells and these respective mixed cells are cultured in the media optimal to each culture cell.

In every tissue culture n-L suspension is used as the control and are cultured with each of tissue culture cells as in the experimental groups. For one series of tissue culture, 16×10^5/ml of lymph-node cells and 4×10^4/ml of tissue culture cells are mixed and 100 u/ml of penicillin are added making the final volume to 10 ml. 1.5 ml of this mixture in each series are pipetted to six short test tubes and cultured.

The culture method is the replicate cell culture of EVANS *et al.*[3] and the culture is conducted at 37° C. In Experiment 1 the cell counts are taken of 3 test tubes each at culture time 29 and 52 hours; in Experiments 2 and 3 at the culture time 24 and 48 hours. In the cell counting processes, the medium is decanted, crystal violet solution is added, and after incubating for 30 minutes at 37°C, the cells attached to the tube wall are gently detatched by a rubber cleaner, and a uniform cell suspension is prepared by a gentle stirring. A droplet of the cell suspension is placed on Bürker-Türk hemocytometer and the cell counts are taken more than six times for each test tube and the average of three test tubes is taken as the increase in the number of the target cells.

RESULTS

Experiment 1 : It was found that both the regional lymph-node cells (iso-L) obtained from C3H female mouse after isotransplantation of the skin and those cells (MC-L) obtained from the C3H mouse after the isotransplantation of MC-induced sarcoma did not inhibit the proliferation of A cells (Fig. 1).

Taking the number of A cells at the time of addition of n-L (control) as 100 per cent, at the culture hour 52, iso-L cells seem to have inhibited the prolifera-

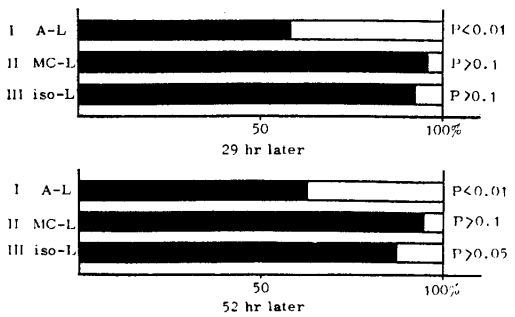

Fig. 1 Percentage of inhibitory effect of A-L, MC-L and iso-L cells on A strain cells

Note: ☐ This column denotes the percentage of inhibition.
I denotes A-L +A strain cells, II denotes MC-L +A strain cells, and III denotes iso-L + A strain cells.
Percentage of increase in the number of culture cells is expressed as $\frac{\text{the number of culture cells at the addition of regional lymph-node cells}}{\text{the number of culture cells at the addition of normal lymph-node cells}} \times 100$.
For the method, see text.

tion of A cells about 87 per cent but on comparing 65 per cent inhibition shown by A-L, there is hardly any significant difference observable.

Experiment 2 : Everyone of the lymph-node cells (B-L), from the mice positive to the tuberculin test and c-L, and i-L, do not inhibit the growth of A cells (Fig. 2).

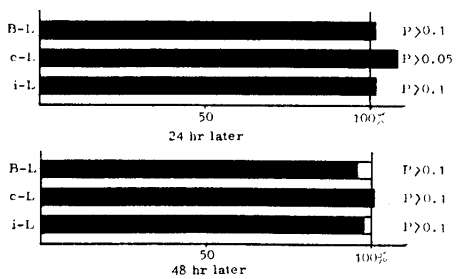

Fig. 2 Percentage of inhibitory effect of B-L, c-L, and i-L cells on A strain cells

Note: ☐ This column denotes the percentage of inhibition.
B-L : The lymph-node cells from the C3H mouse positive to the tuberculin test.
c-L : The lymph-node cells from the C3H mouse 3 weeks after the injection of complete adjuvant.
i-L : The lymph-node cells from the C3H mouse 3 weeks after the injection of incomplete adjuvant.
For the method, see text.

Experiment 3 : The regional lymph-node cells (A-L) obtained 10 days after the isotransplantation of A cells show the inhibitory effect on the proliferation of A cells as high as 40～60 percent, and on those homologous cells such as M cells and JTC-11 cells likewise by 30～40 per cent. On the other hand, these regional lymph-node cells show hardly any inhibitory effect on the growth of L cells, fibroblasts derived from C3H mouse and heterologous cells such as AH-66F and HeLa-S_3 cells (Fig. 3).

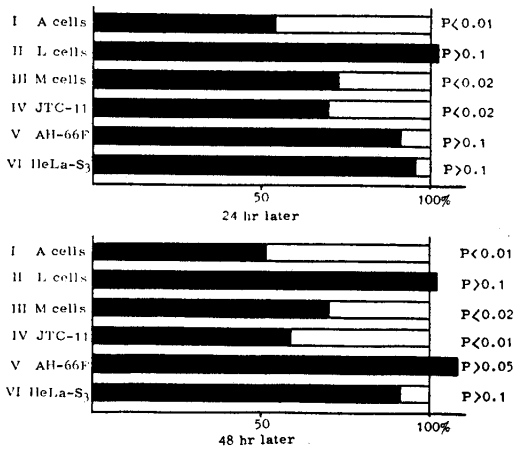

Fig. 3 Percentage of inhibition on the proliferation of I-VI culture cells by regional lymph-node cells obtained 10 days after the isotransplantation of A strain cells
Note: ☐ This column denotes the percentage of inhibition. For the method, see text.

DISCUSSION

From the fact that the regional lymph-node cells (A-L) from isologous C3H mouse transplanted with A cells derived from C3H mouse mammary cancer apparently inhibit the proliferation of A cells in tissue culture, there arises a question what factor or factors are responsible for this inhibitory effect. The first reason for it seems to be that there is a difference in histocompatibility gene and hence it is possible for C3H mouse to recognize A cells as not-self. Looking at the results of Experiment 1 in both isografting of skin in full thickness and the isotransplantation of MC-induced sarsoma to C3H mice, the regional lymph-node cells from these mice do not inhibit the growth of A cells in tissue culture, and further, in the mutual exchange of skin graft between C3H mice the skin graft survives permanently on each animal[4] and this means that A cells have the same histocompatibility as C3H mouse. Consequently, the inhibitory effect

of A-L cells on A cell seems not to be caused by the difference in histocompatibility gene. Another question is whether or not those lymph-node cells obtained from the animal in the state of delayed hypersensitivity would show specifically anti-tumor activity *in vitro*. There are reports[5] that *in vivo* the animal treated with BCG shows anti-tumor activity against transplanted cancer. As can be seen from the results in Experiment 2, those lymph-node cells (B-L) from the mouse proven positive to the tuberculin test as well as c-L and i-L cells all do not inhibit the proliferation of A cells in tissue culture. Therefore, the second question would not support anti-tumor activity of A-L cells. Supposing the first and second questions do not stand for reasoning, then there comes another problem that A-L cells might induce the action of specific antigen (lacking in C3H mouse) of A cells and cause anti-tumor activity. As observed in Experiment 3, A-L cells do not at all inhibit the proliferation of heterologous cells like AH-66F and HeLa-S_3 cells, and also isologous L cells, but they do inhibit A cells as well as homologous JTC-11 and M cells. This means that in the interaction *in vitro* between A-L and A cells there seems to be at least species specificity. Furthermore, considering that everyone of A cell, JTC-11 cell and M cell is the mammary cancer cell derived from different strains of mouse and that isologous MC-L cell has no effect on A cell, it is assumed that A-L cells act on a specific antigen common in mouse mammary cancer. In Experiment 3, the fact A-L inhibits most markedly A cells suggests that A-L, aside from the antigen common in mammary cancer, acts also on specific antigen of A cell. In other words, A-L cells seem to be sensitized specifically by A cells.

BRONDZ[6] conducted transplantation of Sa-I (benzpyrene-induced mouse spindle cell sarcoma) and MX 5 (methylcholanthrene-induced sarcoma) to coisogenic pair of mice that differ only in H-2 locus, and to coisogenic pair of mice that differ in weak antigen other than H-2 locus either subcutaneously or intraperitoneally and conducted the mixed tissue culture of lymphoid cells from these animals and cancer cells, and found that only in the case where the donor cell and the host cell differed in H-2 antigen, the donor cell is destroyed by the lymphoid cells.

ROSENAU[7] likewise have studied the effect of the sensitized lymphoid cells from BALB/c mice inoculated with L cells, spleen cells of C3H mice or C_{57}BL mice on various cell lines *in vitro*. As the result cell lines derived from the same mouse strain as the cells used for sensitization are lysed by the sensitized lymphoid cells but cell lines derived from other mouse strains or heterologous cells in general are not destroyed. It means that lysis of donor cells occurs in the interaction between the cells having different H-2 antigen.

TAYLOR[8] states that the spleen cells from mouse or guinea pig sensitized with L cells show a marked cytolytic effect on L cells as well as on fibroblasts of the

sensitized guinea pig, and KOPROWSKI[9] reports that the lymph-node cells of the rat sensitized with guinea-pig spinal cord specifically destroy glial cells from the brain tissue of newborn puppy but do not damage fibroblasts, meaning that each case demonstrates organ specificity.

Judging from the findings of the *in vitro* experiments so far mentioned and from the result of Klein's experiment[10,11] showing that the resistance against methylcholanthrene-induced sarcoma in the authochthonous mice is located in the lymph-node cells of host, A-L cells seem to act specifically on cancer antigen *in vitro*.

SUMMARY

For purpose to study specificity in the growth inhibition effect of sensitized lymph-node cells on target cells, the regional lymph-node cells obtained from the truly isologous mouse previously inoculated with A strain cells (derived from C3H mouse mammary cancer) were cultured with A cells in various ways, and obtained the following results.

1. Those regional lymph-node cells from the isologous mice transplanted with the skin of C3H mouse or MC-induced sarcoma do not inhibit the growth of A cells in tissue culture.

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From these results it is assumed that the sensitized regional lymph-node cells act specifically on cancer antigen.

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